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In vitro effect of cholesterol on calcifying activity of vesicles isolated from rabbit aortas

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Abstract

It has been shown that vesicles play a key role in the onset mechanism of aortic calcification related to cholesterol-induced atherosclerosis. This study using a rabbit model was conducted to determine whether cholesterol exerts a direct effect on vesicle's calcifiability. Inclusion of cholesterol in calcifying media stimulated ATP-initiated deposition of calcium in a dose-dependent manner by vesicles isolated from normal aortas using crude collagenase digestion. By contrast, cholesterol did not significantly affect ATP-promoted calcification if vesicles were isolated from atherosclerotic aortas. To determine whether high cholesterol levels in atherosclerotic vesicle preparations may have already maximized calcifying activity and therefore account for lack of the vesicle's response to the sterol, Fourier transform infrared spectroscopy (FT-IR) was used to compare the cholesterol contents in control and atherosclerotic vesicles. The spectral patterns revealed higher levels of cholesterol in vesicle preparations from atherosclerotic aortas than those from normal aortas. Removal of extra-vesicular cholesterol micelles from atherosclerotic vesicles by a relatively low centrifugal force sensitized the vesicles to cholesterol stimulation causing a 2-fold increase in calcifying activity. Of various oxidized forms of cholesterol tested, 7-keto and 6-keto cholesterol enhanced the activity by 2-fold. Altogether, these observations suggest that cholesterol and especially its oxidized forms may induce aortic calcification by directly enhancing the vesicle's ability to calcify.

Keywords: Cholesterol; Calcification; ATP; Aorta; Calcifying vesicle

1. Introduction

Dystrophic calcification in aortas or arteries is a reliable predictor for cardiovascular diseases. Despite its implications in coronary artery disease, myocardial infarction, and aortic ruptures, clinical significance of aortic calcification that may lead to stabilizing or hypercoagulable situation remains uncertain [1]. Numerous studies using animal models indicated that a role for dietary cholesterol in the induction of aortic calcification [2–4]. However, the mechanism whereby cholesterol induces aortic calcification remains unclear. The co-localization of cholesterol and mineral in atherosclerotic lesions in human aortas has been shown by Hirsch et al. [5]. Brown et al. [6] have shown the accumulation of cholesterol, 25 hydroxycholesterol, and 7-keto-cholesterol in atheromatous lesions. These observations suggest that cholesterol may have a direct effect on mineral formation. We have previously demonstrated that the accumulation of calcifying vesicles preceded histologically identifiable mineralization, indicating a key role for vesicles in initiating calcification [4,7]. However, whether cholesterol or its oxidized forms may directly exert a stimulatory effect on calcifying activity of vesicles has not been investigated. This study provides the first evidence that cholesterol and its oxidized derivatives can directly enhance ATP-promoted vesicle calcification.

2. Experimental procedures

2.1. Induction of atherosclerotic calcification by a high cholesterol diet

Eight rabbits of 4-month-old juvenile rabbits were fed a standard rabbit chow supplemented with 0.5% cholesterol and 2% peanut oil (supplied by Harland Teklad). After 6 months of chronic dietary interventions, rabbits developed conspicuous calcification in the intimal areas adjacent to the

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media [4,7]. The degrees of atherosclerosis and calcification were assessed by histology using hematoxylin and eosin (H&E) and alizarin red staining procedures, respectively.

2.2. Isolation of calcifiable vesicles from aortas

Calcifying vesicles were isolated from atherosclerotic aortas using the method of Hsu et al. [7]. Eight segments of ascending thoracic aortas (3 in. long) were collected and the adventitia including attached adipose tissues were removed, and immediately submerged in cold phosphate-buffered saline. The segments were minced into fine pieces, washed once with 10 ml PBS by centrifugation, and then digested for 3 h at 37 °C in a solution (15 ml/g of tissue) containing 0.1% of crude collagenase (Boehringer Mannheim, Type B), 0.25 M sucrose, 0.12 M NaCl, 0.01 M KCl, 100 U/ml of penicillin, 1 mg/ml of streptomycin, and 0.02 M Tes buffer (*N*-tris[hydroxmethyl]-methyl-2-amino-ethanesulfonic acid), pH 7.45. Normal aortas under the similar digestion were used as controls. The digests were centrifuged at $800 \times g$ to precipitate cells and cell debris. The supernatants were then centrifuged at $30{,}000 \times g$ for 10 min to precipitate mitochondria and microsomes. The resultant supernatants were centrifuged at $300{,}000 \times g$ for 20 min. The pellets were resuspended in TBS (10 mM Tris-buffered saline, pH 7.6)-0.25 M sucrose and centrifuged. The resultant precipitates were then resuspended in 1 ml TBS-0.25 M sucrose to yield a protein concentration of about 0.3–1 mg/ml. Some of the vesicle preparations were pooled.

2.3. Calcium deposition

To measure Ca deposition, the method of Hsu et al. [7] was used with a slight modification. The calcifying medium was prepared as the following. A basal medium consisted of 50 mM Tris, pH 7.6 (37 °C) 85 mM NaCl, 15 mM KCl, 1 mM $MgCl_2$, and 1.45 mM $CaCl_2$. Note that pH was either adjusted to 7.6 at 37 °C or to pH 7.9 at 25 °C. Aliquots of various concentrated stock solutions were added separately to 2-fold concentrated basal media to reach a final concentration of 30 mM $NaHCO_3$, 2.3 mM Pi, and 1 mM ATP, pH 7.5. Small aliquots of water were added to a final volume of 100 μl. These immediate arrangements before the incubation served to minimize spontaneous formation of calcium phosphate, calcium bicarbonate, and unwanted increases in pH levels due to bicarbonate hydrolysis. The reaction was then initiated by the addition of aliquots of calcifying vesicles to the mixture to obtain a final protein concentration of 20 μg protein/ml. The mixture (100 μl) was incubated for 5 h at 37 °C in a humidified incubation chamber under atmospheric CO_2. $^{45}Ca^{2+}$ (1×10^6 cpm) was used as tracer. At the end of incubation, the reaction mixtures were filtered through 0.1 μ pore-size Durapore membranes (Millipore) attached to a Millipore vacuum trapping device. The membrane filters were washed twice each with 1 ml of Tris-buffered saline, pH 7.6 (TBS) and then transferred to vials containing scintillation fluids for radioactivity counting. The nonspecific $^{45}Ca^{2+}$ deposition is defined as the radioactivity nonspecifically bound to the filters under the identical conditions in the absence of calcifiable vesicles ($0.6 \pm 0.2\%$ of the total radioactivity). These nonspecific counts were then subtracted from the radioactivity in the presence of calcifiable vesicles with or without ATP under various experimental conditions. Ca deposition is expressed as "nmol Ca/ml calcifying media/5 h" and is calculated using the following formula: [(cpm with calcifiable vesicles minus nonspecific binding)/total cpm], multiplied by the concentration of $CaCl_2$. Transmission electron microscopy, calcifying activity, and the ATPase activity measurements indicated that no significant amounts of vesicles and mineral passed through filters although some size ranges of mineral and vesicles could be smaller than the pore size of the filters (not shown).

2.4. Fourier transform infrared spectroscopic (FT-IR) assessments of cholesterol in vesicles

FT-IR was used to estimate the content of cholesterol in vesicle preparations of atherosclerotic aortas [4]. Vesicles isolated from normal aortas as a result of collagenase

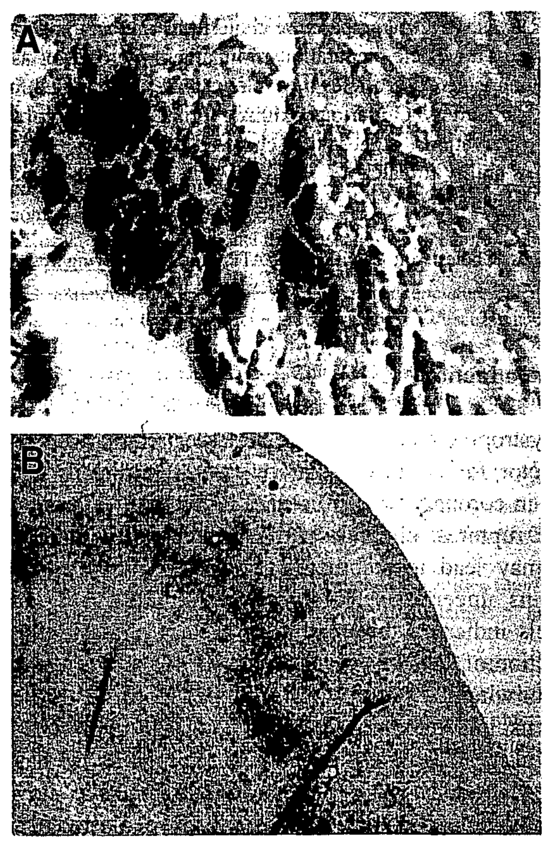

Fig. 1. Induction of aortic calcification by a cholesterol-enriched diet. Rabbits were fed 0.25% cholesterol and 2% peanut oil for 6 months. Histological assessments demonstrated focal calcification in the intima adjacent to the media. (A) H&E staining. The dark blue stains represent heavily calcified areas. (B) Alizarin red staining. The red stains represent heavily calcified areas. Magnification: 100 ×.

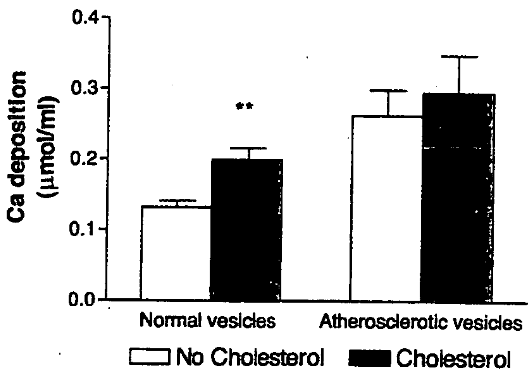

Fig. 2. Effect of cholesterol on Ca deposition by vesicles isolated from normal and atherosclerotic aortas. Vesicles (100 μg protein/ml) were exposed to calcifying media with and without cholesterol (500μg /ml). The activity was expressed as nmol ^{45}Ca deposited per ml of calcifying media. Values are means ± S.E. from five respective vesicle preparations. Total vesicle proteins are also calculated. Paired t-test is used to assess statistical significance. Double asterisks were assigned to those data significantly differed from the respective zero cholesterol control ($P < 0.05$).

digestion were used as controls. Vesicles (0.1 mg protein) were centrifuged at 300,000 × g for 30 min and the resulting precipitates were washed twice with water by the centrifugation and resuspension steps. The washed precipitates were suspended in 0.3% KBr and lyophilized. Pure cholesterol suspended in KBr was used as reference standard. Two major peaks at 1458 and 1383 obtained from pure cholesterol were used as references for characteristic peaks for the identification of cholesterol in the vesicle samples.

2.5. *Effect of cholesterol and its oxidized derivatives on vesicle calcification*

Since cholesterol and its oxidized derivatives are soluble in pyridine, various concentrations of the lipid sterols were prepared in pure pyridine (HPLC grade). A 1-μl aliquot of various cholesterol concentrations was added to calcifying media and followed by the addition of calcifying vesicles. The media were adjusted with water to reach a final volume of 100 μl. The control blank included calcifying media containing 1% pyridine.

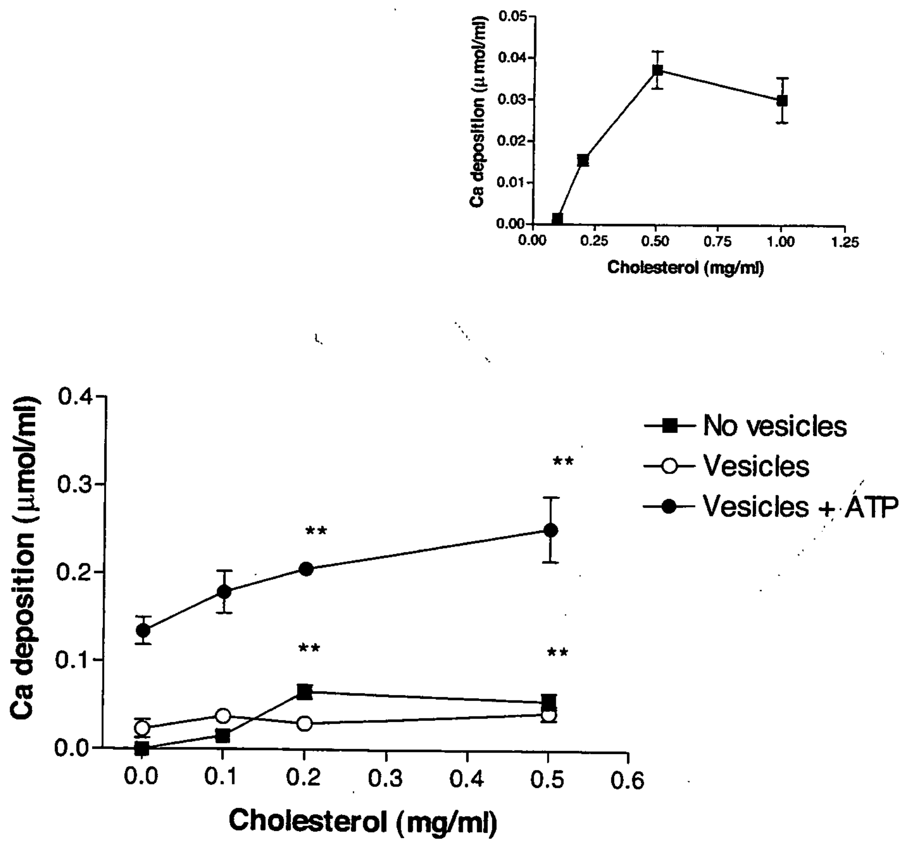

Fig. 3. Effect of cholesterol concentrations on Ca deposition initiated by vesicles isolated from normal aortas. Vesicles (100 μg protein/ml) from three normal aortas were exposed to calcifying media with various concentrations of cholesterol in the presence and absence of 1 mM ATP. Double asterisks were assigned to those data significantly differed from the respective zero cholesterol control ($P < 0.05$). The expanded scale of cholesterol-induced Ca deposition in the absence of vesicles in a separate experiment is shown in the inset. The one-way ANOVA analysis indicates the differences among various concentrations are significant ($P < 0.01$). Cholesterol at 1 mg/ml was not considered since it reaches the maximal solubility under the defined conditions of calcifying media. The activity is expressed as nmol ^{45}Ca deposited per ml of calcifying media. Values are means ± S.E.

2.6. Protein and phosphate assays

The inorganic orthophosphate and protein concentrations were determined by the methods of Martin and Doty [8] and a BioRad kit, respectively.

3. Results

3.1. Induction of atherosclerotic calcification

Four-month-old juvenile rabbits fed a diet supplemented with 0.25% cholesterol and 1% peanut oil for 6 months developed pronounced atherosclerosis with aortic calcification in the lesions as indicated by histological assessments using H&E and alizarin red staining procedures (Fig. 1). Calcification occurred predominantly in the intimal area adjacent to the media.

3.2. Effect of cholesterol on vesicle calcification

Fig. 2 shows that vesicles isolated from atherosclerotic aortas were more active in ATP-initiated Ca deposition than those from normal control aortas. The inclusion of 0.5 mg/ml of cholesterol in calcifying media significantly stimulated ATP-initiated Ca deposition by 55% in the presence of control vesicles isolated from normal aortas by crude collagenase digestion ($P<0.05$). In contrast, cholesterol did not have a significant effect on Ca deposition if calcifying vesicles were isolated from atherosclerotic aortas (Fig. 2, $P>0.05$). The cholesterol effect was dose-dependent (Fig. 3). An attempt to increase cholesterol concentrations beyond 0.5 mg/ml to further enhance calcification was not feasible because of its limited solubility in calcifying media. In the absence of vesicles, cholesterol (0.1–0.5 mg/ml) caused a small deposition of calcium (Fig. 3, inset). One-way ANOVA analysis indicates that differences among various cholesterol concentrations (0.1–0.5 mg/ml) were highly significant ($P<0.01$). Cholesterol in excess of 0.5 mg/ml has reached its solubility limits in calcifying media and thus did not further enhance Ca deposition. The degree of Ca deposition by cholesterol alone was close to that by vesicles but much below ATP-promoted Ca deposition by vesicles.

3.3. FT-IR spectroscopic assessments of cholesterol content in calcifying vesicles

To determine whether insensitivity of atherosclerotic vesicles to cholesterol was due to high levels of preexisting cholesterol, cholesterol in vesicle preparations was estimated by FT-IR spectroscopy. After subtracting the normal vesicle spectra from those of atherosclerotic vesicles, the presence of two distinct peaks at 1458 and 1383 wavelengths characteristic spectra of cholesterol revealed higher levels of cholesterol in vesicles from atherosclerotic aortas than those isolated from the normal tissue (Fig. 4).

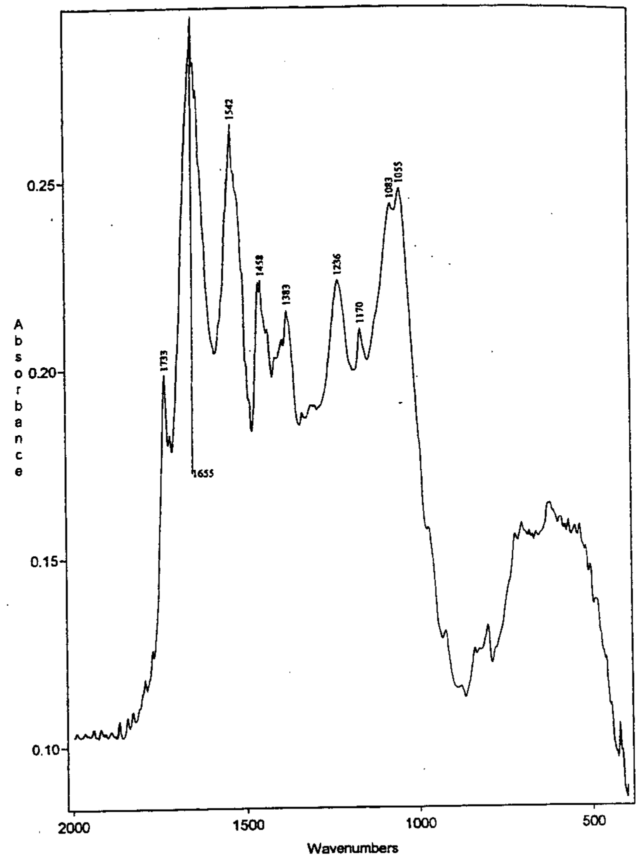

Fig. 4. Infrared spectroscopic estimates of cholesterol content in vesicles isolated from atherosclerotic aortas. The spectrum was obtained by subtracting absorption spectra displayed by control vesicles from those of atherosclerotic vesicles. The presence of two spectral peaks at 1458 and 1383, characteristic of cholesterol absorption, revealed a higher amount of cholesterol in the atherosclerotic vesicles than that in the control vesicles.

3.4. Effect of removal of cholesterol micelles from atherosclerotic vesicles preparations on calcification

It is plausible that the presence of cholesterol micelles in vesicle prepared from atherosclerotic aortas could have already maximized the activity and thereby may account for the lack of stimulation on Ca deposition by cholesterol. To test this possibility, pyridine was used to remove cholesterol micelles from the vesicle preparations and followed by assessing calcifying activity. Aliquots of vesicle preparations were extracted with pyridine at a pyridine/protein ratio (w/w) of 0.5 and then subjected to centrifugation in a microfuge at top speed. Supernatants, which contained cholesterol and lipids, were discarded and pellets were washed repeatedly with TBS buffer using centrifugation. The resulting precipitates were resuspended in a small aliquot of TBS-0.25 M sucrose. For the control, pyridine was replaced by water.

In the absence of ATP, the addition of 0.5 mg/ml of cholesterol did not affect calcifying activity of the water-treated vesicles, which were used as controls. In contrast, cholesterol enhanced the activity of pyridine-insoluble pre-

cipitate fractions (Fig. 5). At variance with the promoting effect of ATP on vesicle calcification, 1 mM ATP inhibited Ca deposition by the precipitate fraction from pyridine extraction. Since ATP is known to inhibit mineral formation because of its strong affinity for calcium [9], pyridine extraction could have inactivated ATPase, which is necessary for the removal of inhibitory ATP. To test this hypothesis, the effect of pyridine extraction on ATPase activity was assessed. Pyridine treatment was found to completely inhibit ATP hydrolysis (data not shown). In contrast with the whole atherosclerotic vesicles, ATP-dependent calcifying activity of the control precipitates obtained by subjecting the same vesicles to water instead of pyridine was markedly stimulated by the addition of 0.5 mg/ml of cholesterol (Fig. 5, the last white column).

3.5. *Effect of various cholesterol derivatives on vesicle calcification*

Various oxidized and hydroxyl forms of cholesterol were shown to be present in lesions [6] and thereby, like cholesterol, may also stimulate aortic calcification through their effects on vesicle's ability to calcify. To test this hypothesis, the effect of various oxidized derivatives of cholesterol on vesicle calcification was investigated. As shown in Fig. 6, 25 hydroxyl-cholesterol (5-cholestene-3 β, 25-diol) exerted a small but significant stimulation on ATP-initiated calcification by 20% ($P<0.01$). Both 7-keto-cholesterol (5-cholesten-3β-ol-7-one) and 6-keto-cholesterol enhanced the

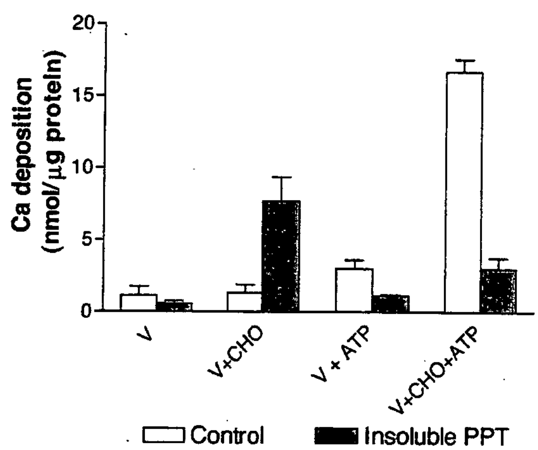

Fig. 5. Effect of removal of cholesterol micelles from atherosclerotic vesicles preparations on calcification: to remove cholesterol micelles, aliquots of vesicle preparations were extracted with pyridine at a pyridine/protein ratio (w/w) of 0.5. The extracts were then centrifuged in a microfuge at the top speed and resulting pellets were resuspended in pyridine and followed by the removal of residual cholesterol using centrifugation. The pellets were washed repeatedly with water to remove pyridine by resuspension and centrifugation steps. The final pellets were resuspended in an aliquot of TBS containing 0.25 M sucrose. For control experiments, pyridine was replaced by the same volume of water. Values are means ± S.E. from six respective vesicle preparations. Double asterisks were assigned to those data significantly differed from the respective zero cholesterol control ($P<0.05$).

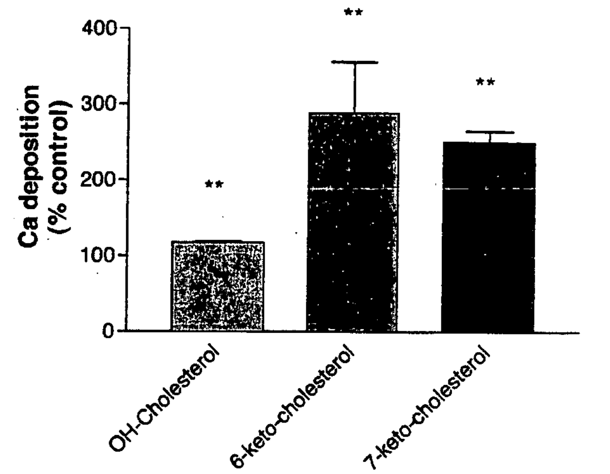

Fig. 6. Effect of various oxidized or hydroxyl forms of cholesterol on vesicle calcification. Various cholesterol oxides were dissolved in pyridine and 1-µl aliquots of the solution were then added to 100 µl calcifying media to reach a final cholesterol concentration of 0.5 mg/ml. Vesicles were then added to calcifying media in the presence or absence of ATP to initiate calcification. The extent of stimulatory effect of various oxidized cholesterols is expressed as the percentage of control Ca depositing activity in the presence of 1% pyridine, which did not inhibit Ca deposition. Values are expressed as means ± S.E. from three respective vesicle preparations. Paired *t*-test is used to calculate statistical significance. Double asterisks were assigned to those data significantly differed from the respective zero cholesterol control ($P<0.05$).

activity by 2-fold and therefore were more effective than hydroxycholesterol. The dose–response patterns of vesicle calcification to each oxidized derivatives were similar to cholesterol data (data not shown).

4. Discussion

The initiation of dystrophic calcification in atherosclerotic aortas appears to be an extremely complex process implicating initial cellular events and subsequent matrix readiness for mineral deposition (see Ref. [10] for review). Recent studies using rabbits demonstrated that accumulation of calcifying vesicles precedes aortic calcification as a result of high cholesterol diets [4,7]. The present study clearly shows that cholesterol directly enhanced the ability of vesicles to calcify, providing additional mechanisms of cholesterol-induced aortic calcification. The ability of cholesterol to enhance Ca deposition by vesicles from normal but not by those from atherosclerotic aortas further supports the contention that accumulation of cholesterol may underlie vesicle-initiated aortic calcification. Thus, both increased production [4,7] and activation of calcifying vesicles reported here may play a key role in the onset of dystrophic calcification.

Although cholesterol alone induced Ca deposition in the absence of vesicles, the level of cholesterol-induced calcification was unable to reach that of ATP-promoted vesicle calcification, suggesting that ATP may potentiate the cho-

lesterol effect. The ability of cholesterol to stimulate vesicle calcification was limited by its insolubility in calcifying media under the defined experimental conditions. Whether some cholesterol carriers such as lipoproteins of various densities in extracellular fluids may conceivably increase cholesterol solubility to stimulate calcification remains to be determined.

The inactivation of vesicle calcification by pyridine extraction suggests either the denaturation or removal of the vesicle's essential components such as ATPase is responsible for the inhibition. Indeed, the addition of ATP further diminished ATP-independent calcium deposition by the pyridine-insoluble fraction. The diminished vesicle calcification apparently was attributable to the irreversible inactivation of ATPase, thus causing the accumulation of ATP, a potent inhibitor of mineralization [9]. These observations reveal a dual role of ATP in the process of biological calcification.

The molecular mechanism for the differences in the responses of whole vesicles and fractionated vesicles from atherosclerotic aortas to cholesterol treatment is not known. The comparison of cholesterol contents between normal and atherosclerotic vesicles by FT-IR analysis (Fig. 4) suggests that relatively low-speed centrifugation ($12{,}000 \times g$) may have removed lighter cholesterol micelles from the atherosclerotic vesicle preparations. As a result, the cholesterol-depleted vesicles became responsive to cholesterol treatment as in the case of normal vesicles. Altogether, these observations further support the contention that cholesterol may exert its effect on aortic calcification through a direct stimulatory effect on vesicle calcification.

Several oxidative forms of cholesterol accumulate in atheromatous lesions of rabbits as a result of cholesterol dietary interventions [11]. Oxidized forms of cholesterol have been shown to play an important role in atherogenesis [6]. However, whether these cholesterol derivatives have an effect on aortic calcification has not been established. Cholesterol has been shown to be closely associated with mineral deposits in atherosclerotic lesions [5]. Watson et al. [12] demonstrated that hydroxycholesterol stimulates vascular cell-mediated calcification in vitro. These findings and the present study suggest that dystrophic calcification may be mediated through the direct stimulatory effect of cholesterol on both cell- and vesicle-mediated calcification. It is interesting to note that 7- and 5-ketocholesterols were more potent than hydroxycholesterol in their ability to stimulate vesicle calcification. The molecular mechanisms whereby various oxidized cholesterol stimulate vesicle calcification is currently under investigation.

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